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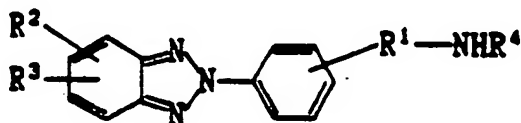
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54 BENZOTRIAZOLE DERIVATIVES AND REAGENTS CONTAINING SAME FOR ANALYZING CARBOXYLIC ACIDS.

57 The invention provides compounds represented by formula (I) and their salts, wherein R¹ represents C₁-C₅ alkylene, R² and R³, which may be the same or different, each represents hydrogen, C₁-C₅ alkyl, C₁-C₅ alkyloxy, amino, mono- or di(C₁-C₅ alkyl) amino (provided that R² and R³ do not represent hydrogen at the same time) or, when taken together, represent C₁-C₅ alkylendioxy, and R⁴ represents hydrogen or C₁-C₅ alkyl. Since the compounds react with a carboxylic acid in a short time at room temperature in the presence of a condensing agent to produce amides emitting strong fluorescence, thus being useful as reagents for determining carboxylic acids rapidly with high sensitivity.



(I)

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BENZOTRIAZOLÉ DERIVATIVES AND REAGENTS FOR CARBOXYLIC ACIDS THEREOF

FIELD OF THE INVENTION

Carboxylic acids exist widely in the living body as the metabolites of carbohydrates, lipides, and proteins, and each acid plays important parts. This invention relates to the compounds suitable for microanalysis of these carboxylic acids and to fluorescence labeling reagents thereof.

BRIEF EXPLANATION OF THE DRAWINGS

Fig 1 illustrates the relationship between the concentration of flufenamic acid and the peak area of the chromatogram, where the ordinate shows the peak area ($\times 10^4$) and the abscissa the concentration of flufenamic acid (ng/500ul).

Fig 2 is the chromatogram obtained in Experiment Example 1, where the abscissa shows retention time (minute) and the peak indicated by the arrow corresponds to flufenamic acid (about 0.2ng).

Fig 3 illustrates the relationship between the concentration of ibuprofen and the peak area ($\times 10^6$) of the chromatogram, where the ordinate shows the peak area ($\times 10^6$) and the abscissa the concentration of ibuprofen.

Fig 4 is the chromatogram obtained in Experiment Example 2, where the abscissa shows retention time (minute) and the peak indicated by the arrow corresponds to ibuprofen (about 0.02ng).

BACKGROUND OF THE INVENTION

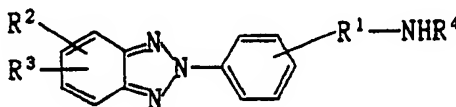
Recently, attention has been paid to prostaglandins with a wide variety of physiological activities, bile acids metabolized from cholesterol and oxy acids, keto acids, etc which are thought important for the diagnosis or the treatment of congenital dysbolism, so it is hoped to develop for the method for quantitative detection of them with simplicity, rapidity, and high sensitivity.

Mr. Nimura et al disclose 9-anthryldiazomethane (N. Nimura, T. Kinoshita: Anal. Lett., 13, 191 (1980)). 9-anthryldiazomethane is advantageous in its reactivity with carboxylic acids at room temperature, but often causes problems in the course of detection because of its unstability.

Mr. Tsuchiya et al disclose the method for fluorescence detection of 7-hydroxy-4-hydroxymethylcoumarin, prepared by labeling carboxylic acids with 7-acetoxy-4-bromomethylcoumarin (ABMC), separating the same by reversed phase HPLC followed by alkaline hydrolysis (H. Tsuchiya et al; J. Chromatogr., 234, 121 (1982)). This method has a merit that the detection limit is high (several ten fmol) without the influence of the solvent in the mobile phase, but some demerits that it needs complicated procedures such as heating in the case of the reaction of carboxylic acids with ABMC, and alkaline hydrolysis after separation.

DISCLOSURE OF THE INVENTION

In view of the circumstances above, the present inventors have tried to develop labeling reagents which are reactive under mild conditions and have highly sensitive but do not disturb the detection and completed the present invention. The compounds, which the present invention provides, of the following formula:



wherein R^1 means C_1 - C_5 alkylene; R^2 and R^3 each is identically or differently hydrogen, C_1 - C_5 alkyl, C_1 - C_5 alkyloxy, amino, or mono- or di- C_1 - C_5 alkylamino, except that R^2 and R^3 are hydrogen at the same time, or

form together C₁-C₅ alkylendioxy; and R⁴ means hydrogen or C₁-C₅ alkyl, or a salt thereof (hereafter referred to simply as the compounds of this invention) react with carboxylic acids quickly at room temperature in the presence of a condensing reagent. The reactants are amides having strong fluorescence and excessive reagents can be eliminated easily. Thus, the compounds of this invention make the detection

5 of carboxylic acids, simple, rapid, very sensitive, and therefore they are useful.

The compounds of this invention react with carboxylic acids in an aprotic solvent in the presence of a condensing reagent and a base or an acid-scavenger in place of the base, within about 30 minutes at room temperature, thereby corresponding amides showing strong fluorescence are formed. Besides excessive reagents can be removed easily from the reaction mixture by passing the reaction solution through a

10 silicagel column for the pretreatment. The compounds of this invention react with a wide variety of carboxylic acids, regardless of aromatic or aliphatic ones. It is desirable that R² and R³ have higher electron-donating property, since such property gives high fluorescence intensity to the resulting compound.

The compounds of this invention can be prepared easily by known reactions in the field of organic

15 chemistry. For example, as shown below, substituted aniline (a) is subjected to known diazo coupling reaction with diazo compound (b) having an aminoalkyl group protected by a suitable protective group (Step 1), then to oxidative ring-closing reaction (Step 2), followed by deprotection (Step 3), to give the compound (I) of this invention.

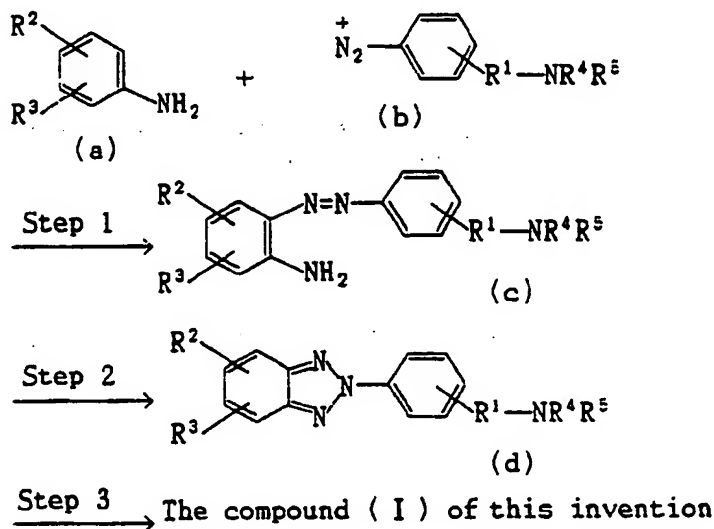
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In the reaction scheme, R¹, R², R³, and R⁴ each has the same meaning as defined above, R⁵ means a protective group.

The process above are explained below step by step. (Step 1)

45 The reaction is usually carried out in an aqueous solution at a temperature from ice-cooling to 15° C. Aniline derivative corresponding to the diazo compound (b) is dissolved or suspended in an inorganic acid, which is then reacted with nitrous acids such as sodium nitrite to obtain the highly reactive diazo compound (b). Sulfamic acid or urea is added thereto to remove excessive nitrous acids.

Next, the substituted aniline compound (a) is added thereto, whereby it is easily coupled with the diazo compound (b) to give the compound (c). The reaction is completed in several minutes to several hours.

(Step 2)

The azo group of the compound (c) easily forms an oxidative ring closure with the adjacent amine to give the triazole compound (d). The reaction is completed in several minutes to several ten hours at room temperature or under heating, if carried out in a water-containing organic solvent in the presence of a

55 copper-ammonia complex. (Step 3)

The compound (d) is subjected to hydrolysis in a water-containing organic solvent in the presence of an acid or a base to give the compound (I) of this invention. The reaction is completed in several minutes to several days at room temperature or under heating.

In the invention, C₁-C₅ alkylene means a straight chain C₁-C₅ alkylene including methylene, ethylene, propylene, butylene, and pentylen, especially methylene or ethylene is preferable. C₁-C₅ alkyl means a straight or branched chain C₁-C₅ alkyl, including methyl, ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, and tert-pentyl, etc.

As for the protecting group, all the known protecting groups for amine can be used. Among others, those stable for acids are preferable, for example, acetyl, trifluoroacetyl, and benzoyl, etc.

The representative examples of the compounds of this invention are listed below, which do not limit the scope of the invention.

- (1) 2-(p-aminomethylphenyl)-N,N-dimethyl-2H-benzotriazolyl-5-amine,
 - (2) 2-[p-(2-aminoethyl)phenyl]-N,N-dimethyl-2H-benzotriazolyl-5-amine dihydrochloride,
 - (3) 2-(p-aminomethylphenyl)-2H-benzotriazolyl-5-amine,
 - (4) p-(5,6-dimethoxy-2H-benzotriazol-2-yl)benzylamine,
 - (5) 2-[p-(5,6-methylenedioxy-2H-benzotriazol-2-yl)]phenethylamine,
 - (6) 4-[m-(5-propoxy-2H-benzotriazol-2-yl)phenyl]butylamine,
 - (7) 4-butoxy-2-[(3-m-methylaminopropyl)phenyl]-2H-benzotriazolyl-5-amine,
 - (8) N-butyl-5-[o-(5-ethoxy-7-butyl-2H-benzotriazol-2-yl)phenyl]pentylamine,
 - (9) N-isopropyl-2-[m-(5,6-ethylenedioxy-2H-benzotriazol-2-yl)]phenethylamine, and
 - (10) 2-(o-aminomethylphenyl)-N,N-dimethyl-2H-benzotriazolyl-5, 6-diamine.
- This invention is further explained in the following Examples and Experiments, which are not intended to limit the scope of this invention.

Example

The compounds 1-5 of this invention were prepared as shown in the following Examples 1-5. Physical constants on each compound are shown on table 1.

Example 1

Preparation of 2-(p-aminomethylphenyl)-N,N-dimethyl-2H-benzotriazolyl-5-amine (1)

To a solution of N-(p-aminobenzyl)acetamide (6.6g) dissolved in 100ml of 10% hydrochloric acid was added 10% sodium nitrite (30ml), while being stirred under ice-cooling. Fifteen minutes later, 10% ammonium sulfamate (50ml) was added thereto, and the mixture was stirred for 15 minutes. The reaction mixture was adjusted to about pH 5 with sodium acetate, added with N,N-dimethyl-m-phenylenediamine dihydrochloride (8.4g), and the resulting mixture was stirred for 4 hours.

The reaction mixture was adjusted to about pH 9 with 1N sodium hydroxide, filtered to collect precipitates, which were then washed well with water. They were recrystallized from dimethylformamide/methanol to give the pure azo compound as red plates, m.p. 138-139° C.

To a solution of the resulting azo compound (8.7g) dissolved in pyridine (100ml) was added an ammoniacal cupric sulfate solution which was prepared by dissolving cupric sulfate pentahydrate in 150ml of 14% aqueous ammonia, then the mixture was refluxed for 4 hours. After completion of the reaction, the mixture was cooled and extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulfate and evaporated to leave a residue, which was recrystallized from aqueous ethanol to give the objective compound (1) as acetate, yellow needles, m.p. 196-197° C. To a solution of the acetate (7g) dissolved in ethanol (200ml) was added 10% hydrochloric acid (50ml), then the mixture was refluxed for 5 days. After completion of the reaction, the reaction mixture was concentrated to about 50ml (total volume), and filtered. The filtrate was neutralized with sodium bicarbonate to leave precipitates, which were collected by filtration, washed well with water, and recrystallized from methanol to give the objective compound (1).

Example 2

Preparation of 2-[p-(2-aminoethyl)phenyl]-N,N-dimethyl-2H-benzotriazolyl-5-amine dihydrochloride (2)

The reaction was performed according to the same manner as in Example 1 except that N-(p-aminophenethyl)-2,2,2-trifluoroacetamide hydrochloride (8.1g) was used in place of N-(p-aminobenzyl)-acetamide. The extract which was resulted from an oxidative ring closure reaction with an ammoniacal cupric sulfate solution were dissolved, without recrystallization, in ethanol (50ml), combined with 5N aqueous solution of sodium hydroxide (20ml), and the mixture refluxed for 2 hours. After completion of the reaction, the mixture was combined with water (100ml) and extracted with ethyl acetate. The ethyl acetate layer was evaporated under reduced pressure and the oily residue was dissolved in ethyl ether (20ml). After filtration, the filtrate was added to an ethanolic solution of hydrochloric acid to give the objective compound (2).

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Example 3

15 Preparation of 2-(p-aminomethylphenyl)-2H-benzotriazol-5-amine (3)

The reaction was performed according to the same manner as in Example 1, except that m-phenylenediamine dihydrochloride (7.2g) was used in place of N,N-dimethyl-m-phenylenediamine dihydrochloride, to give the objective compound (3).

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Example 4

25 Preparation of p-(5,6-dimethoxy-2H-benzotriazol-2-yl) benzylamine (4)

The reaction was performed according to the same manner as in Example 1, except that 3,4-dimethoxyaniline (6.4g) was used in place of N,N-dimethyl-m-phenylenediamine dihydrochloride, to give the objective compound (4).

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Example 5

35 Preparation of 2-[p-(5,6-methylenedioxy-2H-benzotriazol-2-yl)]phenethylamine (5)

The reaction was performed according to the same manner as in Example 2, except that 3,4-methylenedioxyaniline (5.6g) was used in place of N,N-dimethyl-m-phenylenediamine dihydrochloride. After the completion of hydrolysis, the reaction mixture was extracted with ethyl acetate, and the organic layer was evaporated under reduced pressure to leave a residue, which was recrystallized from methanol to give the objective compound (5).

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Table 1

| No | Appearance IR (Nujol) | m. p. (°C) | Molecular Formula | Elementary Analysis (Calcd./Found) | | |
|----|--|------------|---|---------------------------------------|--------------|----------------|
| | | | | C | H | N |
| 1 | yellow needles 3300, 3380 | 127-128 | $C_{15}H_{17}N_5$ | 67.39 67.45 | 6.41 6.37 | 26.20 26.15 |
| 2 | light red plates 2450, 2550 | 250-260 | $C_{15}H_{21}N_5Cl_2$ | 54.24 53.58 | 5.97 5.96 | 19.77 19.52 |
| 3 | yellow plates 3150, 3300, 3350, 3430 | 206-207 | $C_{15}H_{13}N_5$ | 65.25 65.27 | 5.48 5.51 | 29.27 29.18 |
| 4 | colorless needles 3300, 3350, 3480 | 155-156 | $C_{15}H_{18}N_4O_2 \cdot$ $\frac{1}{2}H_2O$ | 61.42 61.54 | 5.84 5.83 | 19.10 19.05 |
| 5 | colorless needles 1713, 3230, | 145-147 | $C_{15}H_{14}N_4O_2 \cdot$ $\frac{1}{4}H_2O$ | 62.82 62.66 | 5.10 5.21 | 19.54 19.55 |

Experiment (General Procedures for Analysis)

In a 10ml vial, 0.5ml of an acetonitrile solution of a carboxylic acid compound is placed and acetonitrile solutions of 2-bromo-1-ethylpyridinium tetrafluoroborate (3.7mM), 9-methyl-3, 4-dihydro-2H-pyrido[1,2-a]-pyrimidin-2-one (2.7mM), and the compound of this invention (3.7mM), are added thereto by 50 μ l each and the mixture stirred for about 10 seconds. This solution is allowed to stand at room temperature for about 30 minutes.

This solution (0.5ml) is passed through a silicagel column for the pretreatment, with acetonitrile (1.5ml) as an eluent. Acetonitrile is added to the eluent to make the whole 2.5ml, 20 μ l of which is subjected to HPLC. Excitation wavelength and fluorescence wavelength on each reagent in acetonitrile are shown in Table 2.

Experiment 1 (The compound 1 of this invention as reagent)

Acetonitrile solutions (0.5ml each) containing 3, 6, 15, 30, or 60ng of flufenamic acid were placed into 10ml vials. To each vial were added acetonitrile solutions (50 μ l each) of 2-bromo-1-ethylpyridinium tetrafluoroborate (3.7mM), 9-methyl-3, 4-dihydro-2H-pyrido[1,2-a]pyrimidin-2-one (2.7mM), and the compound of this invention (3.7mM), and the mixture were stirred for about 10 seconds. These were allowed to stand at room temperature for about 30 minutes.

Each of these solution (0.5ml) was passed through a Bond Elut column (silicagel) and eluted with acetonitrile (1.5ml). The total amount of the eluate was made 2.5ml with acetonitrile, the resulting solution (20 μ l) was subjected to HPLC.

[Conditions for Measurement]

Apparatus: Shimadzu LC-4A Pump (Shimadzu) Shimadzu RF-535 Fluorescence HPLC Monitor
(same as above)
Column: Novapack C₁₈ (3.9X150mm; Waters)
Guard Column: Nucleosil 5C₁₈ (4.0x30mm; Nagel)
Mobile Phase: acetonitrile/water = 2/1
Flow Rate: 1.2ml/minute
Wavelength: 395nm for excitation, 510nm for fluorescence emission
[Results]

The relationship between the concentration of flufenamic acid and the peak area of the chromatogram as obtained in the experiment above is shown in Figure 1. The chromatogram is shown in Figure 2.

Experiment 2 (The compound 5 of this invention as reagent)

The experiment was performed in the same way as Example 1, except that the compound 5 of this invention was used as a reagent to acetonitrile solutions (0.5ml each) containing 0.6, 3, 6, 15, 30, or 60ng of ibuprofen.

[Conditions for Measurement]

Apparatus: same as Example 1
Column: Nucleosil 5C₁₈ (4.6x150mm; Nagel)
Guard Column: same as Example 1
Mobile Phase: acetonitrile/water = 5/3
Flow Rate: 1.0ml/minute
Wavelength: 333nm for excitation, 375nm for fluorescence emission
[Results]

The relationship between the concentration of ibuprofen and the peak area of the chromatogram as obtained in the experiment above is shown in Figure 3. The chromatogram is shown in Figure 4.

Experiment 3

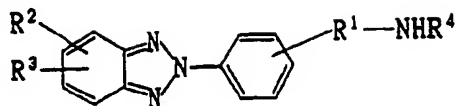
Table 2 shows both excitation and fluorescence wavelength of amide compounds in acetonitrile, which are the reactants of the compounds (1)-(5) of this invention with benzoic acid. The date of detection limit for each amide compound by HPLC is also shown.

Table 2

| No | Excitation Wavelength(nm) | Fluorescence Wavelength(nm) | Detection Limit(fmol) |
|----|------------------------------|--------------------------------|--------------------------|
| 1 | 390 | 488 | 15 |
| 2 | 384 | 486 | 15 |
| 3 | 366 | 456 | 6 |
| 4 | 333 | 370 | 0.5 |
| 5 | 333 | 372 | 0.5 |

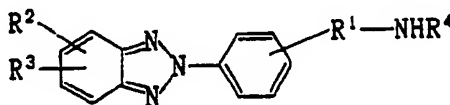
Claims

1. A compound of the following formula:



wherein R¹ means C₁-C₅ alkylene; R² and R³ each is identically or differently hydrogen, C₁-C₅ alkyl, C₁-C₅ alkyloxy, amino, or mono- or di-C₁-C₅ alkylamino, except that R² and R³ are hydrogen at the same time, or taken together may form C₁-C₅ alkylenedioxy; and R⁴ means hydrogen or C₁-C₅ alkyl, or the salt thereof.

2. A reagent for determining carboxylic acids, comprising containing a compound of the following formula:



wherein R¹ means C₁-C₅ alkylene; R² and R³ each is identically or differently hydrogen, C₁-C₅ alkyl, C₁-C₅ alkyloxy, amino, or mono- or di-C₁-C₅ alkylamino, except that R² and R³ are hydrogen at the same time, or taken together may form C₁-C₅ alkylenedioxy; and R⁴ means hydrogen or C₁-C₅ alkyl, or the salt thereof.

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Fig 1

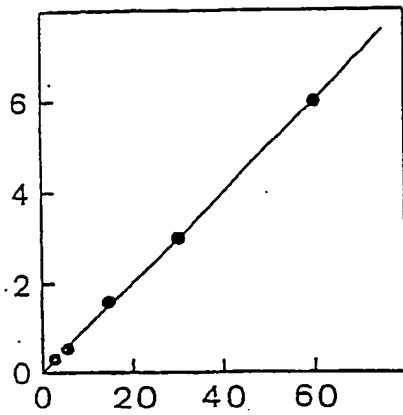


Fig 2

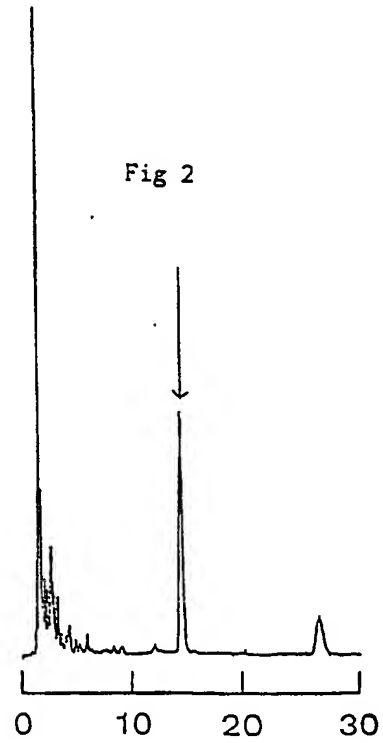


Fig 3

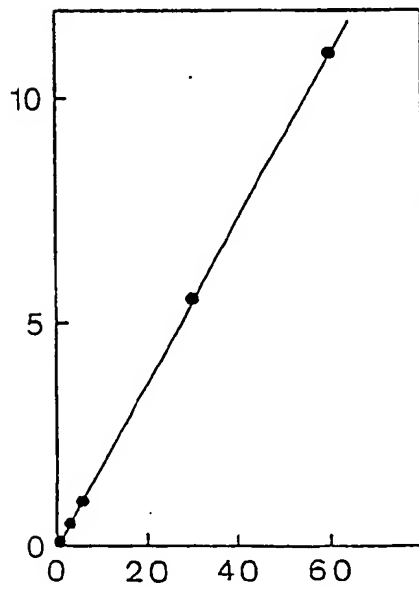
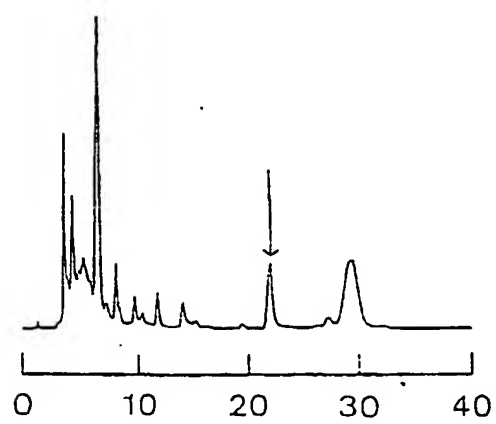


Fig 4



INTERNATIONAL SEARCH REPORT

International Application No PCT/JP89/00519

| | | |
|--|--|---|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl ⁴ C07D249/20, 491/056, G01N33/50, 33/58 | | |
| II. FIELDS SEARCHED Minimum Documentation Searched ⁷ Classification System Classification Symbols IPC C07D249/20, 491/056, G01N33/50, 33/58 Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ | | |
| Category ¹⁰ | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| A | Analystical Letters, Vol. 13, (1980), P.191 | 2 |
| A | Journal of Chromatography, Vol. 234, (1982), P.121 | 2 |
| * Special categories of cited documents: ¹⁴ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "G" document member of the same patent family | | |
| IV. CERTIFICATION | | |
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